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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A01N 63/02, C12R 1/01, 1/38, 1/385, 1/39 // (C12R 1/01, 1:38, 1:385, 1:39)

A1

(11) International Publication Number: WO 99/22598

(43) International Publication Date: 14 May 1999 (14.05.99)

(21) International Application Number: PCT/GB98/03254

(22) International Filing Date: 30 October 1998 (30.10.98)

(30) Priority Data:

9722960.3	30 October 1997 (30.10.97)	GB
9723226.8	3 November 1997 (03.11.97)	GB

(71) Applicant *(for all designated States except US)*: THE UNIVERSITY OF READING [GB/GB]; Whiteknights, P.O. Box 217, Reading RG6 6AH (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: HAGUE, Nigel, Graham, Mackenzie [GB/GB]; 19 Greenleas Avenue, Emmer Green, Reading, Berkshire RG4 8TA (GB). ELAWAD, Sami, Abdulrahaman [SD/GB]; Sibly Hall, Redhatch Drive, Reading, Berkshire RG2 5QW (GB).

(74) Agents: O'BRIEN, Caroline, J. et al.; Mewburn Ellis, York House, 23 Kingsway, London WC2B 6HP (GB).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: BIOPESTICIDE: MATERIALS AND METHODS

(57) Abstract

The invention provides biopesticides for the control of insect pests and/or plant parasitic nematodes. The effective agent is a motile species of bacteria or a bacterial symbiont of an entomopathogenic nematode.

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BIOPESTICIDE: MATERIALS AND METHODS

The present invention concerns materials and methods relating to biopesticides. In particular, the present invention concerns materials and methods relating to biopesticides comprising bacteria.

The damage done to plants and humans by harmful insects and/or plant parasitic nematodes is enormous and their control is mainly achieved through use of synthetic chemical pesticides.

However there are many problems related to the use of chemical pesticides including resistance and toxicity to non-target species. These and other problems and issues create a political and social climate opposed to them. Therefore microbial alternatives including entomopathogenic nematodes have been sought. These nematodes, of the genera *Steinernema* and *Heterorhabditis*, are widespread and have been isolated on every inhabited continent and on many islands. Commercialisation of such nematodes are exempt from registration requirements in many countries. Efficacy has been demonstrated in various markets, so product and sales have begun on a commercial scale.

Steinernematid and *heterorhabditid* nematodes are symbiotically associated with bacteria in the genera *Xenorhabdus* and *Photorhabdus* respectively. The free-living, infective third stage Dauer juvenile of the nematode, which does not feed, carries the bacterium

within its intestine. The infective-stage locates insects, initiates infection and is the only stage in the nematode's life cycle that survives outside the insect in the soil. The infective-stage enters insects via mouth, anus or spiracles and penetrates mechanically into the body cavity where it releases the bacterium. In certain insects, *heterorhabditids* can enter into the insect body cavity through the cuticle. The bacterium proliferates, causes septicemic death of the insect within 24-72 hours and establishes favourable conditions for nematode reproduction by providing nutrients and inhibiting the growth of many foreign micro-organisms. The nematodes feed on multiplying bacteria and dead host tissue, passing through several generations. Eventually, Dauer Juveniles (DJs), carrying symbiont bacteria in their gut, emerge from the insect cadaver. At 18-28°C, the nematodes complete their life cycle in most insects in 8-20 days.

Exposure of insect hosts to high concentrations of *steinernematids* and *heterorhabditids* under laboratory conditions has indicated that the host range includes most insects and at high concentrations extends also to other invertebrates (*Gastropoda, Symphyla, Arachnida, Crustacea* and *Diplopoda*). However, behavioural and ecological barriers have restricted the effectiveness of nematodes to certain soil inhabiting insects. Consequently, laboratory experimental infections do not always appear to translate to the field where the nematodes infect fewer species.

In order to achieve field results comparable to that of standard chemical pesticides the strategy for application of entomopathogenic nematodes has to be very carefully controlled.

Further the current cost of preparing entomopathogenic nematodes for pest control is 10-60% higher than chemical insecticides and some nematodes of interest e.g. heterorhabditid nematodes cannot yet be produced efficiently in liquid fermenters.

Microbial pesticides other than entomopathogenic nematodes have been used and Table 1 summarises the major commercially available microbial insecticides (from Lacey, L.A. and Goettel M.S., 1995 Entomophaga 40, p3-27 and Georgis R 1997 BCPC Symposium Proceedings No 68 p 243-252).

Of the microbes referred to in Table 1, the bacteria *Bacillus thuringiensis* (Bt) is most widely used. Various subspecies which are larvicidal for different host ranges have been introduced. The effective Bt larvicidal agent comprises a toxin and Bt genes that encode toxin production have been manipulated using both recombinant and non-recombinant methods. Manipulation of these Bt toxin genes has enabled their incorporation into crop plants.

However in the case of Bt-transgenic plants, constant control of insect pests will be an extremely strong selective force for insects that are resistant to Bt, necessitating a need for aggressive resistance

management plans where growers set aside acres that are either not treated with insecticides or not treated with Bt.

Owing to the problems of development of resistance there is a real economic need for as diverse a range of biopesticides as possible.

The present inventors have now unexpectedly discovered that isolates of the symbiotic bacteria can be used to control insect pests and/or plant parasitic nematodes.

Thus the present invention provides a new biopesticide composition for the control of insect pests and/or plant parasitic nematodes which comprises as an effective agent a bacterial species which is motile.

The present invention provides a new biopesticide composition which comprises as an effective agent a bacterial species which is flagellate.

The present invention provides a new biopesticide composition which comprises as an effective agent a bacterial species which enters the target insect species via a route other than the alimentary canal. It may enter via the spiracles.

The present invention provides a new insecticide composition which comprises as an effective agent a bacterial symbiont of an entomopathogenic nematode. The bacterial symbiont may be motile. It may be flagellate. It may enter a target insect species via a route other than the alimentary canal. It may enter via the

spiracles.

The present invention provides a new biopesticide which comprises a bacterial species which produces one or more toxins which interfere with the behaviour and activity of a plant parasitic nematode.

The bacterial species may be selected from the genera *Xenorhabdus, Photorhabdus, Pseudomonas* or *Flavimonas*.

The bacterial species may be of the genus *Xenorhabdus* or the genus *Flavimonas*. The bacterial species may be of the genus *Flavimonas*.

The genus *Flavimonas* contains only one species to date *F. oryzihabitans*. The genus was split-off "taxonomically" from the genus *Pseudomonas*. Thus the two genera *Flavimonas* and *Pseudomonas* are closely related. *Pseudomonas* has been developed as a biological agent to control fungi. However *Pseudomonas spp*. have not been suggested as agents to control insects and/or plant parasitic nematodes. In relation to use as an agent to control insect pests and/or plant parasitic nematodes, bacteria of the genus *Flavimonas* may have some advantages over bacteria of the genus *Xenorhabdus*. Firstly *Flavimonas* appears to be more active, with better stability in water and resistance to drying.

The bacterial species may be *Xenorhabdus nematophilus* or *Flavimonas oryzihabitans* or *Xenorhabdus bovienii*. The bacterial species may be *Flavimonas oryzihabitans*.

The biopesticide composition as stated above may comprise a plurality of different bacterial species thus a biopesticide composition according to the invention may comprise a plurality of species selected from bacteria of the genera *Xenorhabdus, Photorhabdus, Pseudomonas, Flavimonas*. Thus the biopesticide composition may comprise a plurality of species selected from *Xenorhabdus nematophilus, Flavimonas oryzihabitans* or *Xenorhabdus bovienii*.

The biopesticide composition may be adapted for application as a liquid suspension of bacteria. The biopesticide composition may be adapted for application as an aqueous suspension of bacteria.

The present invention also provides the use of a bacterial species as stated above (i.e. motile bacteria, flagellate bacterial, spiracle-infecting bacteria, selected from the genera *Xenorhabdus, Pseudomonas, Photorhabdus* or *Flavimonas*) in the preparation of a biopesticide composition wherein the bacterial species comprises a biopesticidally effective element of the composition.

The invention also provides a method for the manufacture of a biopesticide composition, characterised in the use, as an essential constituent of said composition of a bacterial species as stated above (i.e. motile bacteria, flagellate bacterial, spiracle-infecting bacteria, selected from the genera *Xenorhabdus, Pseudomonas, Photorhabdus* or *Flavimonas*) in the

preparation of a biopesticide composition wherein the bacterial species comprises a biopesticidally effective element of the composition.

The present invention also provides a method of controlling insect pests and/or plant parasitic nematodes which comprises applying to environment of the pest or parasite, a biopesticide composition as stated above. The method may comprise application of the composition to a crop plant. In particular the biopesticide composition for application may comprise a liquid suspension of bacteria, for example an aqueous suspension.

The suspension may contain about 3-8 x 10^7 bacterial cells per ml. The suspension may contain about 5-7 x 10^7 bacterial cells/ml. The suspension may contain about 4 x 10^7 bacterial cells per ml.

However a skilled person will be readily able to ascertain the most advantageous dosage rate for a given bacteria or combination of bacteria in relation to the control of a particular pest or combination of pests using the teaching of the specification and common general knowledge.

Example 1

Bacteria suitable for use in relation to the present invention can be obtained from commercial sources and depository institutions.

Alternatively they can be isolated from biological sources such as entomopathogenic nematodes. Such nematodes are available from commercial sources and depository institutions. They can also be readily isolated from insect and soil samples.

Suitable bacteria occur in the infective juveniles of the nematodes and in soils throughout the world. They can be isolated from soil by using suitable hosts, such as the wax moth, *Galleria mellonella*, as trap insects.

1. *Xenorhabdus bovienii*

   This bacteria species is associated with the nematode *Steinernema feltiae* and the bacterium can be obtained from MicroBio, Unit 2 Centro, Boundary Way, Hemel Hempstead, Hertfordshire HP2 7SU, who produce *S. feltiae* as the product NEMASYS.

2. *Xenorhabdus nematophilus*

   This bacterial species is associated with the nematode *Steinernema capocapsae* and can be obtained from Thermotrilogy, 7500 Grace Drive, Columbia MD 21044-4098, USA. Thermotrilogy can also supply bacteria from another Steinernematid, *S.riobrave*.

3. *Photorhabdus luminescens*

   This bacterial species can be obtained from MicroBio (see above) who produce the nematode *Heterorhabditis megidis* as NEMASYS H.

4. *Flavimonas oryzihabitans*

The genus *Flavimonas* was first described in a paper by Holmes, B et al (1987) in International Journal of Systematic Bacteriology Vol 37 No 3, p245-250 ("*Chryseomonas luteola* comb. nov. and *Flavimonas oryzihabitans* gen. nov., comb. nov., Pseudemonas-Like Species from Human Clinical Specimens and Formerly Known, respectively as Groups Ve-1 and Ve-2") Strains of *F. oryzihabitans* have been deposited in the National Collection of Type Cultures: L327/81 as NCTC 11851 and E5726 as NCTC 11852. B2524 is available from the American Type Culture Collection as NTCC 35564.

In an earlier reference the bacteria now designated *F. oryzihabitans* was described as *Pseudomonas oryzihabitans* and obtainable from rice paddy field soils and clinical speciments (Kodma K., et al (1985) International Journal of Systematic Bacteriology Vol 35 No 4: "Two New Species of Pseudomonas: F. oryzihabitans Isolated from Rice Paddy and Clinical Speciments and F. luteola Isolated from Clinical Specimens").

More recently F. oryzihabitans has been isolated from the *Steinernematid, S.abbasi*. This is a new association between a bacterium and an entomopathogenic nematode: Elawad, S., et al in Fundam. Appl. Nematol. (1997) 20 p435-442 "Steinernema abbasi sp.n. Nematode:

Steinernematidae from The Sultanate of Oman)" provides details concerning the isolation and characteristics of S.abbasi which comprise a source of *Flavimonas oryzihabitans*.

The following details concern the preparation of a bacterial isolate from nematodes carrying the appropriate bacterial species. Detailed methodologies of relevance are in: "Steinernematid and Heterorhabditid Nematodes: A Handbook of Biology and Techniques by Jennifer L. Woodring and Harry K. Kaya, Southern Cooperative Series Bulletin 331 published August 1988 by the Nematode Subcommittee of the Southern Regional Project S-135 Entomopathogens for Use in Rest-Management Systems, Arkansas Agricultural Experiment Station, Fayetteville, Arkansas.

Bacteria are isolated from the haemocoele of a larva of the greater wax moth, *Galleria mellonella* infected with the bacterium of interest. Briefly the method is as set out below.

1. Late instar larvae of *G.mellonella* are infected with the appropriate Dauer juveniles (DJs) at the rate of approximately 200 DJs per petri-dish lined with a double layer of filter paper.

2. After the death of the larvae they are washed and surface-sterilized with about 70% alcohol in

staining blocks for 5-10 mins.

3. Sterilized larvae are left to dry in a laminar flow cabinet for about 1 min in a sterile petri-dish.

4. The dead larva are opened with sterile scissors and needle in the laminar flow cabinet and then left for about ½ minute.

5. A drop from the oozing haemolymph from the cadaver is streaked with a sterile toothpick onto a previously prepared nutrient both agar (NBTA) made up as follows: 37 g standard agar, 25 mg bromothymol blue, 1000 ml distilled water, 4 ml of 1% filtered 2,3,5 triphenyl-tetrazolium chloride solution.

6. The agar plates are sealed with parafilm and incubated at 28°C in the dark for 24 hr.

7. Single colonies are selected from the culture to grow in NBTA and then used to streak a new plate of NBTA. The reculturing is done to obtain a culture of the bacterium which was uniform in colony morphology.

8. From the pure cultures of the bacterium a single colony is selected and picked out by sterile toothpick and used to inoculate 50 ml of liquid

broth held in glass flasks. The flasks are covered with sterile cotton wool and paper held by a rubber band. The flasks are placed in a shaking incubator for 2 days at 28°C; the shaking being adjusted to 150 rpm.

The concentration of the bacteria in the broth is estimated using a haemocytometer. Extra sterile broth can be used to dilute the concentration of the bacteria in the solution to give the required dose for application.

Example 2

Bacterial suspensions of *Flavimonas oryzihabitans* and *Xenorhabdus nematophilus* were used to investigate control of *Spodoptera exigua* (cutworm).

Investigation A

20 leaves were detached from the cotton plant *Gossypium hirsutum* and put in separate small covered plastic containers containing sterile distilled water so that the leaves remained fresh during the experiment. The small container with the leaf was then put in a larger plastic container and sealed.

Both the top and bottom surfaces of the leaf were sprayed with 1 ml of bacterial suspension comprising 4×10^7 bacterial cells per ml of solution (in 3% (V/V) Tween 80

as emulsifier). Each cotton leaf had a surface area of approximately 59 cm^2 and one 3rd instar larva of *S.exigua* was placed on each leaf after spraying with the bacterial suspension.

The experiment was placed in a controlled temperature room at 28°C.

The control treatment received Tween 80 in distilled water.

Assessment of the number of dead *S.exigua* larvae was done after 72 hours.

The experiment was conducted using (a) *F. oryzihabitans* and (b) *X. nematophilus.*

Investigation B

This experiment was conducted as in Investigation A above except that, after application of the bacterial suspensions, the leaves were all allowed to dry in a laminar flow cabinet prior to the introduction of one *S. exigua* larva per cotton leaf. As in Investigation A the number of dead *S. exigua* larvae was derived after 72 hours.

Investigation C

This experiment was conducted to investigate the effect of the bacteria on *S.exigua* pupae.

55 g of sterile sand was mixed with 15 ml of bacterial suspension comprising 4 x 10^7 bacterial cells per ml of solution (in 3% (V/V) Tween 80) and placed in a petri

dish. 10 pupae of *S. exigua* were buried in the moist sand and the emergence of *S. exigua* adults assessed after 5 days. 4 such experimental dishes were established.

The results are summarised in Table 2.

In moist conditions *F. oryzihabitans* and *X. nematophilus* caused 100% mortality within 72 hours. Mortality of 75% was found in about 48 hours (data not shown). Application of bacteria to sand in which pupae of *S. Exigua* are placed resulted in mortalities in excess of 75%.

The present inventors have also found that if *F. oryzihabitans* or *X. nematophilus* are incorporated in the artificial diet used to rear *S. exigua,* mortalities in excess of 75% resulted (data now shown).

Although both *F. oryzihabitans* and *X. nematophilus* can be kept fully viable in nutrient broths, they eventually lose their viability in sand (*X.nematophilus* loses viability more rapidly than *F.oryzihabitans*).

Example 3

Bacterial solution of *Flavimonas oryzihabitans* and *Xenorhabdus nematophilus* were used to control *Pieris brassicae* (large white butterfly).

The experiment was conducted using larvae as set out in Example 2, Investigation A above).

The results are summarised in Table 3.

Example 4

Bacterial solution of *Flavimonas oryzihabitans* and *Xenorhabdus nematophilus* were used to control *Galleria mellonella* (greater wax moth).

The experiment was conducted using larvae substantially as set out in Example 2, Investigation C above except that 40 test larvae were used and mortality assessment was carried out after 10 days.

The results are summarised in Table 4.

The experimental results given in Table 4 show that when these bacteria are applied to insect larvae and pupae under conditions when the host larvae, pupae and bacteria are kept moist, there is a resultant high insect mortality.

Example 5

Three experiments were conducted to investigate the effect of symbiotic bacteria on juveniles of plant parasitic nematodes.

Bacterial suspensions of *Flavimonas oryzihabitans* and *Xenorhabdus nematophilus* were mixed with nutrient agar to obtain a concentration of 1.6×10^{10} cells/ml. Second stage juveniles (J_2s) of *Meloidogyne javanica* (root-knot nematode) and *Globodera rotochiensis* (potato cyst nematode) were placed on the agar and their movement observed at 30 min; 1h; 2h; 4h; 8h; 16h and 24h. There was no movement of juveniles of either species after 30

min exposure.

In a second experiment, juveniles of *M.javanica* were exposed to concentrations of 1.6×10^{8} and 1.6×10^{10} cells per ml of *F.oryzihabitans* and a concentration of 1.6×10^{10} of *X.nematophilus*. After exposure for 1h, 3h, 6h, 12h, 24h and 7 days the nematode J_2s were transferred to water to observe whether they recovered some movement. Observation for recovery of movement was carried out at 1h, 3h, 6h, 24h and 7 days post transfer to water.

After treatment with *F.oryzihabitans* for 1h and 3h the nematodes showed partial recovery of movement and partial recovery of movement was observed after 1h treatment with *X.nematophilus*. This was observed for both concentrations of *F.oryzihabitans*. Where (a) juveniles were exposed to 1.6×10^{8} cells/ml *F.oryzihabitans* for 6 hours and greater; (b) juveniles were exposed to 1.6×10^{10} cells/ml *F.oryzihabitans* for 6 hours and greater; and (c) juveniles were exposed to 1.6×10^{10} *X.nematophilus* for 3 hours and greater there was no recovery of movement upon transfer to water.

In a third experiment bacterial suspensions of *F.oryzihabitans* (1.6×10^{10} cells/ml) were stored at 7°C for 10 days and then tested against J_2s of *M.javanica*. There was no movement of J_2s after a minimum exposure of 1h.

Table 1

Major commercially available microbial insecticides

Pathogens	Major targeted group
Bacteria	
Bacillus thuringiensis	
Crystal Protein types:	
Cry IA-G, Cry IIA-C	
Cry IIIA-D	Lepidoptera
Crys IVA-D, Cry IIA	Coleoptera, Culicidae, Simuliidae
B. sphaericus	Culicidae
Virus	
Nuclear polyhedorosis viruses	Lepidoptera, Hymenoptera
Grarulosis viruses	Lepidoptera
Fungi	
Beauveria bassiana	Coleoptera, Lepidoptera, Homoptera, Orthoptera
Metarhizium anisopliae	Coleoptera, Lepidoptera, Homoptera, Orthoptera
Verticillium lecanii	Homoptera, thrips
Nematodes	
Steinernema carpocapsae	Lepidoptera, Coleoptera, (Curculionidae, Chrysomelidae)
S. feltiae	Diptera (Sciaridae)
S. riobravis	Lepidoptera, Orthoptera (mole crickets), Coleoptera (Curculionidae)
Heterorhabditis bacteriophora	Lepidoptera, Coleoptera
H. megidis	Coleoptera

Table 2

The mortality of *S. exigua* larvae and pupae after treatment with the bacteria *F. oryzihabitans* and *X. nematophilus*.

Treatment	Mortality		
	Application on leaves which were kept moist	Application followed by drying	Pupae
Flavimonas oryzihabitans	100%	20%	80%
Xenorhabdus nematophilus	100%	10%	80%
Untreated	0%	0%	10%

Table 3

The mortality of *Pieris brassicae* larvae after treatment with the bacteria *F. oryzihabitans* or *X. nematophilus*.

Treatment	Mortality
Flavimonas oryzihabitans	90%
Xenorhabdus nematophilus	70%
Untreated	0%

Table 4

The mortality of *Galleria mellonella* larvae after treatment with the bacteria *F. oryzihabitans* or *X. nematophilus*.

Treatment	Mortality
Flavimonas oryzihabitans	70%
Xenorhabdus nematophilus	60%
Untreated	0%

CLAIMS:

1. A biopesticide for the control of insect pests or plant parasitic nematodes or both, which comprises as an effective agent a species of bacteria which is a symbiont of an entomopathogenic nematode.

2. A biopesticide according to claim 1 wherein the bacteria is a motile species.

3. A biopesticide according to claim 1 or claim 2 wherein the bacterial species is of a genera selected from *Xenorhabdus*, *Photorhabdus*, *Pseudomonas* or *Flavimonas*.

4. A biopesticide according to any one of claims 1 to 3 which is an insecticide.

5. A biopesticide according to any one of claims 1 to 3 which is a vermicide.

6. A biopesticide according to any one of claims 1 to 5 wherein the bacteria is flagellate.

7. A biopesticide according to any one of claims 1 to 6 wherein the bacteria invades insects via a route other than the alimentary canal.

8. A biopesticide according to claim 7 wherein the bacteria invades insects via the spiracles.

9. A biopesticide according to any one of claims 1 to 8 wherein the bacteria produces a toxin which interferes with the behaviour and activity of a plant parasitic nematode.

10. A biopesticide according to any one of claims 1 to 9 wherein the bacterial species is of the genus *Xenorhabdus*.

11. A biopesticide according to any one of claims 1 to 9 wherein the bacterial species is of the genus *Flavimonas*.

12. A biopesticide according to claim 11 which comprises *Flavimonas oryzihabitans*.

13. A biopesticide according to claim 10 which comprises *Xenorhabdus nematophilus*.

14. A biopesticide according to claim 10 which comprises *Xenorhabdus bovienii*.

15. A biopesticide according to any one of claims 1 to 14 which comprises as an effective agent a mixture of bacteria.

16. A biopesticide according to claim 15 which comprises a plurality of bacterial species of genera selected from *Xenorhabdus*, *Photorhabdus*, *Pseudomonas*, *Flavimonas*.

17. A biopesticide according to any one of claims 1 to 16 adapted for application as a liquid suspension of bacteria.

18. A biopesticide according to any one of claims 1 to 17 adapted for application as an aqueous suspension of bacteria.

19. Use of a species of bacteria which is a symbiont of an entomopathogenic nematode in the preparation of a biopesticide for the control of insect pests and/or plant parasitic nematodes.

20. Use according to claim 19 wherein the bacteria is motile species.

21. Use according to claim 19 wherein the bacterial species is of a genera selected from *Xenorhabdus*, *Photorhabdus*, *Pseudomonas* or *Flavimonas*.

22. A method of controlling insect pests or plant parasitic nematodes or both which comprises applying to the environment of the pest or parasite a biopesticide according to any one of claims 1 to 21.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 98/03254

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A01N63/02 C12R1/01 C12R1/38 C12R1/385 C12R1/39
//(C12R1/01,1:38,1:385,1:39)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A01N C12R

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 98 08388 A (MORGAN JAMES ALUN WYNNE ;JARRETT PAUL (GB); ELLIS DEBORAH JUNE (GB) 5 March 1998 see examples 1,3,6 ---	1-10, 13-23
X	US 5 616 318 A (DUDNEY RALPH A) 1 April 1997 see the whole document ---	1-10, 13-23

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

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Date of the actual completion of the international search	Date of mailing of the international search report
9 February 1999	24/02/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Klaver, J

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 98/03254

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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